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BIOCHEMICAL BIOMARKERS AT THE SITE OF INFLAMMATION AND IN PERIPHERAL BLOOD

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Biochemical biomarkers at the site of inflammation and in peripheral blood

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To Viljam

”*Livet har ett värde*
endast genom sitt innehåll för andra”

Dag Hammarskjöld

ABSTRACT

Biochemical biomarkers are small molecular species, naturally varying or experimentally induced, which are measurable in body fluids and which may provide alternative or complementary tools to describe disease processes or to assess responses to pharmacological treatment. The focus of this thesis is inflammatory biomarkers, mediators, chemokines and cytokines that attract immune cells and that regulate the course of the inflammatory process at the site of inflammation. A major objective was to evaluate the behavior of these molecules in blood.

In the first study we used two established animal models, the Freund´s Complete Adjuvant (FCA) model and the Carrageenan model, to induce experimental inflammation in rats and could demonstrate a significant decrease of prostaglandin E_2 (PGE_2) in synovial fluid following treatment with two COX-inhibitors, naproxen and rofecoxib, despite the drugs having limited efficacy on overall joint swelling. L(+)-lactate, the end product of glycolysis, reflects cell activity and was therefore hypothesized to be a suitable novel indicator of experimental inflammation. We found that L(+)-lactate levels were unaffected by COX-inhibitors in this study, indicating that L(+)-lactate might be used as a biochemical biomarker for on-going inflammation in individuals being treated with COX-inhibitors. In the next study we therefore assessed levels of L(+)-lactate, as well as chemokines and cytokines, in joint fluids from human osteoarthritis patients and in an additional animal model, the monoiodo acetate (MIA) model, which primarily induces cartilage degradation, osteophyte formation and mild synovitis. MIA induced distinct inflammatory biomarkers in a biphasic manner, but in considerably lower amounts than FCA, suggesting that the underlying pathology in these two models is significantly different. As expected, L(+)-lactate was readily detectable in synovial fluid in the MIA model and in humans, correlating with the levels of cytokines and chemokines, and thereby indicating the presence of inflammatory cells in the joint cavity. We also observed two common findings between the MIA model and in humans, namely the presence of IL-6 in joint and serum and the lack of IL-1β and TNF in the same matrices. Nevertheless, we found it difficult to identify a single systemic biochemical biomarker that specifically reflects an ongoing local inflammatory process in osteoarthritis, and nor was it possible to identify a specific translational biomarker between the animal models and OA individuals.

In the third study, in rats subjected to spinal cord injury, the main findings were changes in inflammatory biomarkers in serum during the time of treatment with the drug imatinib, a tyrosine kinase inhibitor. Although serum levels of MCP-1 and MIP-3α were increased at day 1 following injury or sham injury, levels remained similar or lower throughout the study up to 7 days whereas MCP-1 and MIP-3α were further increased at 7 days following injury in the imatinib group. It is plausible that our findings will translate to humans and that the SCI individual could be its own control. Nerve Growth Factor (NGF) is released following tissue injury and therefore the aim was to determine whether NGF is released into the synovial fluid in the FCA model. In the last study we reported the successful development of an assay for the measurement of rat NGF in synovial fluid in rats exposed to FCA. We conclude that the usage of biochemical biomarkers has wide application, and may be used as a complementary tool to other readouts for the analysis of inflammatory conditions.

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LIST OF PUBLICATIONS

I. **Finn A**, Oerther SC. Can L(+)-lactate be used as a marker of experimentally induced inflammation in rats? Inflamm Res 2010;59(4):315-21.

II. **Finn A,** Ängeby Möller K, Gustafsson C, Abdelmoaty S, Nordahl G, Ferm M, Svensson C. Influence of model and matrix on cytokine profile in rat and human Rheumatology (Oxford) 2014;53(12):2297-305.

III. Kjell J, **Finn A**, Hao J, Wellfelt K, Josephson K, Svensson CI, Wiesenfeld-Hallin Z, Eriksson U, Abrams M, Olson L. Delayed imatinib treatment for acute spinal cord injury; functional recovery and serum biomarkers. Journal of Neurotrauma (*Submitted and Revised)*.

IV. Schwieler L, Larsson MK, Skogh E, Kegel ME, Orhan F, Abdelmoaty S, **Finn A**, Bhat M, Samuelsson M, Lundberg K, Dahl M-L, Sellgren C, Schuppe-Koistinen I, Svensson CI, Erhardt S, Engberg G. Increased levels of IL-6 in the cerebrospinal fluid of patients with chronic schizophrenia - significance for activation of the kynurenine pathway. J Psych Neurosci 2014 39(6):140126.

V. Ängeby Möller K, Berge O-G, **Finn A**, Stenfors C , Svensson C I. Using gait analysis to assess weight bearing in rats with Freund's complete adjuvant-induced monoarthritis: Improved predictivity while interfering with the cyclooxygenase and nerve growth factor pathways. Eur J Pharmacol 2015;756:75-84.

CONTENTS

LIST OF ABBREVIATIONS

Cg	Carrageenan
COX	Cyclooxygenase
CV	Coefficient of variation
ECL	Electro chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
FCA	Freund´s Complete Adjuvant
IL-	Interleukin
LLOQ	Low limit of quantification
LOD	Limit of detection
MCP-1	Monocyte chemoattractant protein 1
MIA	Monoiodo acetate
MIP-3α	Macrophage inflammatory protein 3α
NGF	Nerve Growth Factor
NSAID	Non steroid anti-inflammatory drug
OA	Osteoarthritis
PGE_2	Prostaglandin E_2
RA	Rheumatoid arthritis
SCI	Spinal Cord Injury
SD	Standard Deviation
SD rats	Sprague Dawley rats
SF	Synovial fluid
TNF	Tumor necrosis factor

1 INTRODUCTION

1.1 General background on biomarkers

The term biomarker and its application were first mentioned in the 1990s and have since been used extensively, especially in the pharmaceutical industry in connection with the development of drugs (Bowsher et al, 2012). There are several definitions of the term biomarker, but the most accepted description, given by the National Institutes of Health (NIH), is as follows *"a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention.*" (Biomarker Definitions Working Group, 2001). Biomarkers can be biochemical, biomechanical or bio-physiological. Biochemical biomarkers are components like small proteins, lipids etc that are experimentally induced or naturally occurring, which are measurable in mammalian body fluids and gives information about what is happening in the body. Soluble biochemical biomarkers are by classification divided in groups corresponding to diagnostic/disease markers, efficacy markers, prognostic markers or markers that reflect other interventions such as induced inflammation. Examples of biomechanical and bio-physiological biomarkers are electrocardiography (ECG), blood pressure, pulse and fever which are measured using equipment such as stethoscopes, watches, thermometers and non-invasive instruments for the evaluation of medical state.

Soluble protein biomarkers are frequently used to diagnose diseases and assess positive or negative responses to medication, both in the clinic and during clinical trials. For example, testing for levels of the protein known as C-reactive protein (CRP) can help doctors to detect the degree of inflammation or infection. An increasing number of patients are affected by diseases caused by inflammation or do suffer from injuries where inflammation are a part of the dysfunctional outcome. Our focus is therefore to identify and validate reliable biochemical biomarkers that may provide as an alternative or complementary tool, which more reliably describe disease processes caused by inflammation, at an early stage.

1.2 Biochemical biomarkers

1.2.1 Biochemical biomarker development process

The optimal soluble biochemical biomarker should be easy to obtain. Therefore biomarkers that can be detected in serum or plasma from blood, urine or saliva are superior to body fluids such as synovial fluid and cerebrospinal fluid, as the access to the latter is limited due to the invasive sampling techniques needed, and the unwillingness of the patients to donate those samples.

On the other hand, there are challenges (due to low levels, lack of appropriate methods etc) when trying to identify soluble markers in peripheral blood that correlates with disease activity at the site of injury (eg. joints, spinal cord, brain,) because a proportional and sufficient diffusion of the marker from the affected site has to take place. Another challenge

is to identify efficacy markers representing the outcome of a specific drug, such as achieving the optimal time points for assessment. Furthermore, insights to which biomarkers that covary between animal models and human disease will facilitate translation of preclinical data to studies aimed at identifying targets for disease and symptom therapies. A biomarker for the efficacy of a drug, preferably present in blood, may further support stratification variables for analysis of outcome in translational studies, due to the knowledge that not all patients are responders to the given drug.

The development of a biochemical marker is a process consisting of 5 overlapping steps (Fig. 1) (Lee JW, 2009). The ultimate final product is a surrogate biomarker that shows so good correlations with the patient's symptoms that it can replace clinical readouts as the primary efficacy endpoint in clinical phase 2 or 3 trials and that to be used in the healthcare regularly.

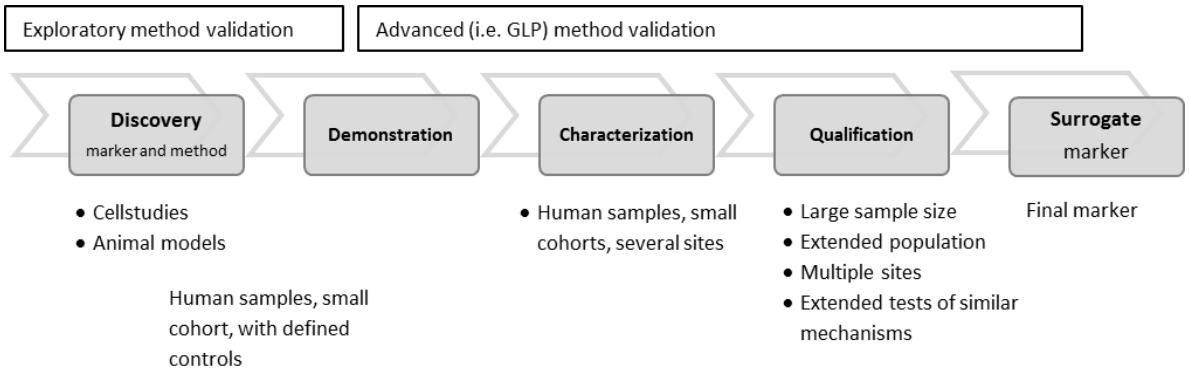

Figure 1. Schematic schedule describing the biomarker development process.
Modified from Lee, JW (2009).

1.2.2 Exploratory method validation

The most common methods and techniques used for the investigation and/or measurement of biochemical biomarkers are immunoassays, based on absorbance (conventional ELISA), electro chemiluminescence (MesoScale Discovery platform) or fluorescence. Other methods useful in the early biomarker discovery are the proximity ligation assay (PLA), electrophoresis (Western Blot), gene expression assays (quantitative real time PCR) and mass spectrometry (Shah et al, 2007). The focus in this thesis is biomarker discovery, and partially also demonstration of some markers. Both methods and inflammatory biomarkers will be presented and discussed from several aspects.

Biomarker level variation

Biomarkers may be expressed at different levels depending on the matrice that is used (DaSilva et al, 2014). It is important to evaluate if it matters if serum or plasma are used for detection of the biochemical markers we explored in this study, especially if they are dependent on clotting factors.

The critical parts for immuno assays are the capture and detection antibodies, they have to be of good quality, be specific for the target and do not cross-react with related proteins. Therefore fit-for–purpose method validations i.e. check for linearity, state the lower limit of quantification and define the minimum required dilution must be performed. Of importance is also stability tests of the marker of interest. Some proteins/cytokines/chemokines degrade fast and do not allow to be thawed several times.

1.2.3 Interpretation of results

In general two criteria are set by the manufacturers when presenting their immunoassay products. The use of the terms limit of detection (LOD), also called sensitivity, and low limit of quantification (LLOQ) needs to be explained. Sensitivity value refers to the lowest measurable value that is statistically not equal to zero (example from R&D systems, ELISAs) while the LLOQ is the lowest concentration at which the coefficient of variation (CV) of the calculated concentration is <20% (or <25) and the recovery of each analyte is within 80% to 120 % of the known value (example from Mesoscale Discovery). The quantitative range of the assay starts at low limit of quantification and ends at the upper limit of quantification. Limit of detection should not be used in other cases than for a yes or no answer. Furthermore, when large series of samples are assayed, it is useful to have a batch of controls for the evaluation of inter-assay variability through the study. Pharmaceutical companies and their biomarker laboratories might have even more defined criteria due to own validation but those numbers above are set by most of the manufacturers.

1.3 Osteoarthritis

Osteoarthritis (OA) is a common joint disease, approximately 25% of the population >45 years are affected in Sweden (Englund et al, 2014). OA is characterized by a mild intra-articular inflammation with limited synovitis, degeneration of articular cartilage, and changes in peri-articular and subchondral bone. OA was traditionally considered to be a "non-inflammatory" arthritis, as it lacks the pronounced systemic and intra-articular inflammatory response that is the key feature of rheumatoid arthritis.

However, signs of inflammatory processes such as increased cell proliferation and synthesis of proteinases, growth factors, cytokines, and other inflammatory mediators by chondrocytes have been detected in early stages of the disease.

Release of IL-1β, TNF and IL-17 have been detected in the OA joint and are thought to contribute to the pathogenesis of OA through several mechanisms, including down regulation of anabolic events and up regulation of-catabolic and inflammatory responses, effects that result in structural damage of the OA joint (Kapoor et al, 2011).

The later stages of OA pathology are thought to be due to excessive wear and tear in the joints. The factors that drive the disease progression are still not completely understood, but e.g. mechanical influences such as load, aging of cartilage matrix, and genetic factors are important. Changes in other cells and tissues of the joint, including the synovium and subchondral bone, are also thought to contribute to pathogenesis (Goldring et al, 2007) (Goldring et al, 2011).

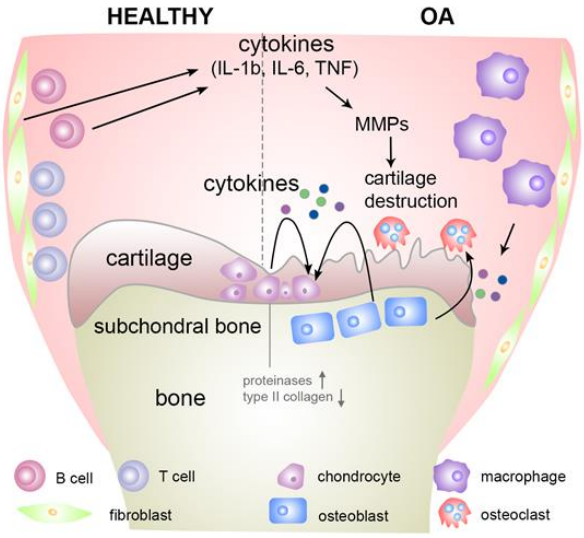

Figure 1. Picture illustrating the proinflammatory cytokines and the cells involved in the pathophysiology of OA. Picture modified from Kapoor et al. Nat. Rev. Rheumatol. 7,33-42 (2011). Courtesy of Jie Su.

Pain is the main reason why these patients seek health-care/medical attention. The disease is chronic by nature and there are no effective disease modifying therapies. Thus, the patients are left to use pharmacologic or alternative therapies to reduce the joint pain and when this is not effective, surgical interventions such as joint-replacement (Kapoor et al, 2011) is performed.

Today the diagnosis of the disease relies on clinical and radiological features and nearly half of patients of osteoarthritis have no symptoms and vice versa. Thus biochemical biomarkers may provide an alternative or complementary tool that more reliably describe disease processes also at an early stage, and concur with the symptoms. Also, the identification of biomarkers that concur with development of OA-like pathology and symptoms in experimental models of OA would facilitate the search for new therapies for disease control and symptom relief. Such factors are searched for by taking e.g. a metabolomics approach (Zhai et al, 2012). Also, intriguing work focused on inflammatory biomarkers is ongoing, but more research is needed, especially when attempting to find soluble biomarkers in the periphery reflecting an on-going disease process in the joint.

1.4 Spinal Cord injury (SCI)

Spinal cord injury affects 12 000 individuals per year in the U.S. and 6 000 to 10 000 individuals (in total) in Sweden have been diagnosed with SCI. There is no drug treatment in use that convincingly shown to has improve the functional recovery after SCI. (www.hjarnfonden.se/diagnoser/ryggmargsskada). Depending on at what level of the spinal cord the injury take place, different types of malfunctions will occur. Cervical lesions may result in tetraplegia while thoracic injuries may result in paraplegia. It is also common that SCI leads to apparent loss of sensation below the injury, and that tissues corresponding to “at-level” and “above-level” become hypersensitive. Another frequent and problem resulting from SCI is the inability to control or empty the bladder, as well as erectile dysfunction, because the nerves regulating these functions descend from the sacral part of the spinal cord. Spinal cord injury progresses in two stages, firstly a damage-causing incidence, after that an extended period when additional loss of cells and nerve fibers will occur and inflammatory and scar forming processes will predominate. During the secondary event there is a possibility that pharmacological intervention may decrease the extent of permanent neurological impairment.

Inflammatory cytokine responses after spinal cord injury have been investigated experimentally and clinically, and may constitute biomarkers for secondary events after spinal cord injury (Krishna et al, 2014, Steeves et al, 2012). Moreover, the drug imatinib has been shown to exert direct effects on the immune system, for example, altering the inflammatory cytokine/chemokine response in serum during treatment of chronic myelogenous leukemia (Adzemovic et al 2013, Hayashi et al, 2012, Pardanani et al, 2003, Seggewiss et al, 2005, Cools et al, 2003).

1.5 Animal models of disease

1.5.1 OA-related joint inflammation and joint pain

Different animal models are utilized in order to not only increase our understanding of the molecular events that occur in joint tissues at the onset and during the progression of OA, but also to delineate the mechanisms that drive OA-induced pain (Gregory et al, 2012, Orita et al, 2011). Examples of common rat models of joint inflammation and/or OA like pathology are: i) the carrageenan model ii) the Freund´s complete adjuvant (FCA) and iii) the monoiodo acetate (MIA) model. The carrageenan model is induced by an intra-articular injection of carrageenan to the joint, which induces an inflammation based on rapid infiltration of granulocytes to the site of injection. This model is relatively short lasting with sign of inflammation and pain lasting for 24-48 hours. The FCA model is established by with an emulsion including adjuvant and a mycobacterial protein, injected intra-articularly. This model causes both joint inflammation and cartilage degradation is more long-lasting (1-3 weeks) compared to the carrageenan model. The MIA model is induced by intra-articular injection of monoiodo acetate, which generates a low-grade long-lasting (weeks) inflammation and cartilage loss (Orita et al, 2011, Guingamp et al, 1997). Subchondral bone is also affected. However, no consensus exists regarding if any of these models are relevant for human OA. Hence, while the different animal models provide information on joint biology and OA pathology, differences between the models also makes comparisons between studies difficult and the translation of animal results to human OA challenging.

1.5.2 SCI

The most common method to subject rats to spinal contusion injury is by using a dedicated instrument called Impactor. (Abrams et al, 2012, Gruner J.A, 1992, Kjell et al, 2013)
The dorsal surface of the spinal cord is exposed by laminectomy of the spinal column (at T9 and the caudal half of T10) and by dropping a 10 g weight from a specified height onto the spinal cord e.g. 12.5 or 25 mm to induce a mild or moderate contusion injury, respectively. Mild injury typically results in all animals being able to support their own weight with the hind limbs, which allows sensory testing and automated locomotor assessment.
A moderate injury results in none to a few animals able to weight support and does not allow the above behavior tests. Furthermore, in contrast to a mild injury, a moderate injury typically results in less variability for the assessed parameters of locomotor recovery and a more robust cytokine response originating from the injured spinal cord. Depending on the readouts the most appropriate model has to be chosen.

1.6 Inflammation

1.6.1 The inflammatory process

Inflammation is the innate immune system response to an infection caused by a pathogen, tissue injury caused by e.g. blunt force, tissue penetration or damaging heat or cold, or exposure to irritants or toxins, which involves the local vascular system, recruitment of leukocytes to the initiating site and release of molecular mediators. The inflammatory process is critical as it protects the body by isolating the damaged area, cause release of mediators such as chemokines and cytokines that attract immune cells and regulate the course of the inflammatory process at site and, in later stages, promote the healing of affected tissues.

Acute inflammation

Acute inflammation can occur within minutes of the injury, but it can also take several hours before the inflammatory response has fully developed. An acute inflammation starts by the movement of plasma proteins and leukocytes cells from the bloodstream into the injured tissues. Release of prostaglandins (PG), histamin and other mediators from local resident inflammatory cells (e.g. macrophages and mast cells) cause blood vessel dilatation, resulting in a local reduction in blood pressure and allows the circulating leukocytes to get in contact with the endothelial cells.

The recruitment of leukocytes to the site of inflammation is a multistep process involving i) attachment of circulating leukocytes to endothelial cells ii) migration through the endothelium iii) penetration into the tissue and iiii) cellular activation and response to the injury or infection. In most forms of acute inflammation, neutrophils, which respond rapidly to chemokines, predominate in the inflammatory infiltrate during the first 6 to 24 hours, after that they are replaced by monocytes/macrophages in 24 to 48 hours. Local release of cytokines, including chemotactic factors, induce a number of responses in leukocytes such as further production of arachidonic acid metabolites e.g. PGE_2, degranulation and secretion of lysosomal enzymes, modulation of leukocyte adhesion molecules and secretion of cytokines.

This process causes the symptoms that are typically associated with inflammation, including redness, swelling and pain, and in some cases increased body temperature and loss of function in the immediate area. When the injury starts to heal or the source of infection has been neutralized, the symptoms of inflammation also resolve. However, if the inflammatory trigger remains active or repeatedly reoccur, the inflammation may develop into a more chronic state (Wold et al, 2007)

Chronic inflammation

Many of the features of acute inflammation continue also in state of chronic inflammation, for example, the blood flow and the capillary permeability are still increased and the

recruitment and accumulation of white blood cells continues. Chronic inflammations is primarily mediated by monocytes and long-lived macrophages and at later stages, other cells, including lymphocytes are recruited to the site of inflammation. Macrophages phagocytize pathogens/irritants at the site of the inflammation and they are important secretory cells releasing inflammatory factors. Thus activated macrophages are the main source of the cytokines that are involved in inflammation, but other leukocytes and mast cells also contribute. Lymphocytes entering the inflamed tissue can serve several roles. For example T-cells activate macrophages in a pathogen/antigen-dependent manner and T-cells can directly kill virus-infected cells. B-cells produce antibodies that specifically target invading microorganisms or tissues for destruction. Repair of the damaged tissue is important for return to homeostasis, and for this the damaged cells are replaced with cells of the same type or with fibrous connective tissue. Chronic inflammation is involved in a number of disease states when the body is unable to repair tissue damage.
(http://course.washington.edu./conj/inflammation/chronicinflam.htm)

The immune cells are dependent on glucose as an energy source to perform the described effector functions, and cortisol increases the glucose levels in blood during inflammation. Increased glycolysis is well-known pathway by which macrophages regenerate their energy stores during phagocytosis and it is also established that aerobic glycolysis contributes to lactate (a pyruvate metabolite) accumulation in the presence of oxygen, especially under inflammatory conditions (Maciver et al, 2008). The level of glucose metabolism can be monitored using non-invasive FDG-PET imaging as a biomarker of the intensity of cellular activity at the sites of inflammation. The level of lactate may be a soluble biochemical biomarker depicting the degree of inflammation, as described in paper I.

1.6.2 Inflammatory mediators

As mentioned above, while an injury, disease or pathogen invasion typically starts the inflammatory process, there are a variety of chemical factors, which affect, stimulate, and control the inflammatory process. The pattern of soluble cytokines and other cell markers expressed will reflect whether a pro-inflammatory or anti-inflammatory reaction is ongoing at the inflammation site. Typical pro-inflammatory markers include, IL-1, IL-6, TNF-α, IL-12, IL-17, IFN-γ, MCP-1, MIP-3α, sICAM-1. Typical anti-inflammatory markers are, but are not limited to, IL-4, IL-10, IL-5 and TGFβ. Some of the factors with more prominent roles are described below.

Prostaglandin E_2 (PGE_2)

Prostaglandins are small-molecule lipid mediators that are derivatives of arachidonic acid. They are produced by two cyclooxygenases (COX), the constitutively active cyclooxygenase COX-1 and the inducible COX-2, followed by the action of different prostaglandin synthases.
At the site of inflammation PGE_2 contributes to local increases in blood flow and therefore starting edema formation by agents such as bradykinin and histamine. Furthermore, PGE2 also promotes inflammatory pain by sensitizing afferent nerve endings.

Agents such as non-steroid anti-inflammatory drugs (NSAIDs) and glucocorticoid steroids represent some of the most common and effective pharmaceutical agents to inhibit or block prostaglandin production. (Giuoli et al 2002, Kalinski et al, 2012).

Nerve growth factor (NGF)

Several studies have shown that under the inflammatory process, there is a local increase of NGF at the site of inflammation (Prencipe et al, 2014). NGF belongs to a family of neurotrophins, a group of proteins that have similar structures and functions. Immune cells express the NGF receptors, tyrosine kinase A (TrkA) and neurotrophin p75 (p75-NTR). TrkA is specific for NGF whereas p75-NTR binds all neurotrophins with equal affinity. Activation of TrkA receptor leads to production and release of pain inducing neuropeptides such as substance P (SP) and calcitonin-gene related peptide (CRRP). NGF also directly sensitizes sensory nerve cells via as pain fibers express TrkA receptor and activates signal transduction. These activated pathways phosphorylate transient receptor potential (TRP) channels, which in turn increases excitability of pain neurons (MacMahon S, 1995, Bracci-Laudiero L, 2010). Of note, increased NGF concentrations have been observed in the synovial fluid of patients with rheumatoid arthritis and in serum of systemic lupus erythematosus (SLE), closely following the progress of the disease (Bracci-Laudiero L, 2010) Further, NGF mRNA expression and protein levels are increased in synovial fluid in animal models of inflammation such as carrageenan-induced or Freund's complete induced adjuvant arthritis.

Cytokines/Chemokines

Interleukins and chemokines such as IL-6, IL-8, IL-1β, TNF, MCP-1 (CCL-2), MIP-3α and the prostaglandin PGE_2 might represent biochemical biomarkers of inflammation and/or pain in OA. As metabolic cell-activity may covary with the inflammatory process in the joint the utility of measuring the pyruvate metabolite L(+)-lactate will add further information about the state of the inflammation.

1.6.3 Why these biochemical biomarkers?

Among the proinflammatory cytokines involved in osteoarhtritis, IL-1β and TNF are considered the major players (Miller et al, 2014, Kapoor et al, 2011, see fig 1, Ru Liu-Bryan et al, 2013) and these are the two most extensively studied. These are produced by chondrocytes, mononuclear cells, osteoblasts and synovial tissues. Furthermore, both IL-1β and TNF are thought to diffuse into synovial fluid and act on chondrocytes to suppress matrix synthesis. They also induce expression of other cytokines such as IL-6 and IL-8, and promote cartilage catabolism by producing matrix-degrading enzymes in both chondrocytes and synoviocytes.

IL-6 is a pluripotent cytokine thought to be a key player in systemic inflammation and arthritis, and on-going discussions and studies explore whereas it is a pain trigger or not.

Previous studies demonstrate that synovial fluid IL-6 levels could help to classify OA patients and may point to a subgroup with a particular impact from their immune system (Svensson CI, 2010, Doss et al, 2007).

MCP-1 (CCL2) is member of the C-C class of the beta chemokine family and mainly involved in the initiation of inflammation, e.g. it is a potent chemoattractant for monocytes and macrophages to the area of inflammation (Deshmane et al, 2009).

MIP-3α, a CC (cysteine cysteine motif) chemokine ligand, CCL20, is chemotactic, not only for dendritic cells but also for (memory) T-cells and studies indicate that MIP-3α and its receptor CCR6 may function to recruit monocytes and memory lymphocytes from the RA peripheral blood into the RA joint (Chabaud et al 2001, Ruth et al, 2003). Recently, it has been confirmed that increased levels of CCL20 are expressed in subchondral bone tissue of rheumatoid arthritis patients (Lisignoli et al, 2007). The same author suggested a differential role of this chemokine in OA and RA regarding proliferative response in osteoblasts (Lisignioli, 2009).

2 AIMS OF STUDIES

- Investigate if L(+)-lactate can be used as a covariat marker for inflammation and/or pain-like behavior in animal models of joint pain.

- Examine if cytokines and chemokines are present in the synovial fluid and blood in animal models of joint pain and investigate the potential of these factors as biochemical biomarkers of inflammation and/or pain-like behavior.

- Examine if L(+)-lactate, cytokines and chemokines as potential biomarkers of pain/inflammation in patients with osteoarthritis.

- Examine if there is a candidate/possible biochemical biomarker of inflammation in spinal cord injury in rats, after and before specific treatment with imatinib.

- Examine if rat Nerve Growth Factor (NGF) is present in the synovial fluid in animal models of joint pain and investigate the potential of these factors as biochemical biomarkers of inflammation and/or pain-like behavior.

3 STUDY DESIGN

Table I. Study design.

Phase	Intervention/model	Paper I	Paper II	Paper III	Paper IV	Paper V
	Rat	Synovial fluid	Synovial fluid/Serum	Serum		Synovial fluid
Discovery	Carrageenan (Cg)	L(+)-lactate, PGE2 w/wo NSAIDs				
	Freund´s Complete Adjuvant (FCA)	L(+)-lactate, PGE2 w/wo NSAIDs	L(+)-lactate (in SF) IL-1β, TNF MIP-3α IL-6 KC/GRO MCP-1 (CCL-2)			NGF
	Monoiodo-acetate (MIA)		L(+)-lactate (in SF) IL-1β, TNF MIP-3α IL-6 KC/GRO MCP-1 (CCL-2)			
	Spinal Cord Injury (SCI) (Uninjured, sham- injured and injured)			MIP-3α KC-GRO IL-6 w/wo Imatinib		
	Human		Synovial fluid/Serum		#	
Demonstration	Osteoarthritis (OA) (with individual treatment) Healthy age matched individuals (only serum)		L(+)-lactate (in SF) IL-1β, TNF MIP-3α IL-6 IL-8 MCP-1 (CCL-2)			

Treatment for the OA individuals was either celecoxib or prednisolone. The biomarkers, Interleukins (IL-s), macrophage inflammatory protein 3α (MIP-3α), monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor (TNF) and L(+)-lactate were assessed either by immune assay or by a colorimetric method. The NSAIDs used were naproxen or rofecoxib, w/wo (= with or without). KC/GRO in rats represents IL-8 in humans. A multiplex (10-plex) was used for the measurements of cytokines and chemokines including INF-γ, IL-1β, TNF, IL-6, IL-2, IL-4, IL-5, IL-10 and IL-13 and KC/GRO (Paper III)

[#] In Paper IV, no formal biomarker discovery was performed, only methodological aspects.

4 MATERIALS AND METHODS

4.1 ETHICS STATEMENT

All animal experiments were carried out in accordance with protocols approved by the Stockholm Ethics Committee for Animal Experiments (Stockholms Norra och Södra Djurförsöksetiska Nämnder, Sweden) with approval numbers S80/04, S120/06, S111/07, S13/09 S87/11, N623/12, (paper I, II and V), N479/11 and N100/13 (paper III).
All human samples (paper II) were collected with written informed consent under the required IRB/ethics approval (Tissue Solutions Ltd.). The research was approved by the regional Swedish ethical board, ethical permit number 2011/743-31/1, paper II, and for paper IV permit number M75-09.

4.2 ANIMAL MODELS

The animals were housed in transparent Macrolon® cages (3–6 rats per cage) with wooden shavings or thick sawdust as bedding, with free access to food (R70, Lactamin AB, Vadstena, Sweden) and tap water. The lighting was controlled with a dark/light cycle 12:12h (or ± 0.5 h for dusk and dawn). The temperature was set to 20 °C and the animals were acclimatized for at least 7 days before onset of the experiments. For the spinal cord injury rats the temperature in the animal room was kept at 24–26°C.

Carrageenan-induced monoarthritis: (Paper I) Adult Sprague-Dawley male rats (B&K Universal, Sollentuna, Sweden), 230 ± 15 g body weight) were anesthetized by 5% isoflurane in air and 50 µL of λ-Cg (7.5 mg/mL in saline; Sigma, Sweden) were injected into the left tibio-tarsal ankle joint from the dorsal side. Naïve (non-injected) rats were used as controls.

FCA-induced monoarthritis: **(Paper I, II and V)** Male Sprague-Dawley rats (B&K Universal, Sollentuna, Sweden, 215 ± 245 g body weight or Harlan Laboratories B.V., Horst, The Netherlands, 225-250 g), were anesthetized by 5% isoflurane in air, and FCA (Sigma; 1 mg/mL; 50 µL) was injected intra-articularly into the left ankle joint and synovial fluid was collected at specific time points up to 14 days post injection. . As control groups naïve rats were used (paper I) or rats that received intra-articular injection of 50 µL saline followed by collection of synovial fluid at 5 hrs (Paper II). Additional 40 Sprague-Dawley male rats (Charles River, Sulzfeld, Germany) were used in a separate study for biochemical analysis of NGF content in synovial fluid. These rats were induced with FCA monoarthritis or injected with saline and synovial fluid was collected 1, 2, 5 and 10 days post FCA injection and 1 and 10 days following saline injection.

MIA-induced monoarthritis: (Paper II) Lewis male rats (Harlan Laboratories B.V., Horst, The Netherlands, body weight 210-250 g, n=8/group) were anesthetized by 5% isoflurane in air and MIA (Sigma; 2 mg in 25 µL saline) was injected intra-articularly into the left knee joint 1,

2, 4, 7, 10, 17, 21, 28 and 35 days prior to termination. At day 1, MIA groups were matched with control groups that were injected with 25 µL saline intra-articularly.

Spinal cord injured animals: (Paper III) Sprague–Dawley female rats (Scanbur, Germany, body weight 200-275 g) were used. Spinal cord injury was performed by a specific instrument (NYU Impactor, Keck Center for Neuroscience) as previously described In brief, rats received analgesics before the surgery and under anesthesia (isoflurane) the dorsal surface of the spinal cord was exposed by laminectomy (sham injury), and by dropping a 10 g weight from a height (12.5 or 25 mm) onto the spinal cord the contusion injury was induced.

4.2.1 Drugs and drug administration

In Paper I the pharmacological effect of orally administered drugs were evaluated. Naproxen (naproxen sodium salt in tap water; Sigma, Sweden) 7.5 or 30 µmol/kg, or rofecoxib (Vioxx, Merck Sharp & Dohme, Sweden), 30 µmol/kg, was administered orally, 2 and 48 hrs post injection of carrageenan and Freund´s Complete Adjuvant, respectively. In paper III Imatinib tablets were grinder into powder and suspended in 0.1 M PBS, brought to 37 °C for 5 min and then centrifuged at 13000 RCF for 2 min. The supernatant was transferred to a new tube and stored at room temperature (max 2 hrs) until administration. Imatinib treatment was given by gavage (250 mg/kg) either 4, 8, or 24 hours after contusion injury and continued daily for 14 days. One third of the daily dose was administered in the morning the remaining dose in the afternoon.

4.2.2 Sample collection

Collection of blood and synovial fluid (FCA and MIA animals)

The rats were anesthetized with isoflurane at different time points after induction of FCA or MIA monoarthritis and blood collected by cardiac puncture was divided into eppendorf tubes, for preparation of serum, or into tubes containing EDTA. The serum samples were left in room temperature approximately 30 min followed by centrifugation at 4°C (4800 x g, 10 min) and EDTA samples were kept on ice prior to centrifugation at 4°C (4800 x g, 10 min). The supernatants were transferred to eppendorf tubes and stored at -80^{o}C until analysis. For the collection of synovial fluid the animals were sacrificed by intra-cardiac injection of 1-2 mL pentobarbitural. The skin above the ankle or the knee joint was cut transversally and the ligament above the joint was punctuated with a scalpel, and the synovial fluid collected by lavage with 25 µL 0.05M EDTA (Sigma, pH 7.5). The lavage was repeated 4 times. The fluid was kept on ice until centrifugation (maximum 1 hour) at 4°C, (3800 x g, 10 min) and the supernatants stored at –80^{o}C until biomarker analysis.

Collection of blood (SCI animals)

Blood from the SCI animals was collected at days 1, 3 and 7 post injury. Rats were put in a cage heated by a heating pad for 15 min prior to blood sampling. Thereafter the animal was moved into a plastic constrainer and 250 µL of blood was collected from the tail vein. The blood was kept at room temperature for 20-30 min prior to centrifugation at 4°C (4800 rpm for 10 min). The supernatant was collected and transferred to – 80 °C prior to analyze.

4.3 Assays

4.3.1 Colorimetric assay

L(+)-Lactate levels were measured by a colorimetric assay (#K607, BioVision, CA, USA). The method described by the manufacturer was slightly modified by converting the concentrations to mmol/L and extending the standard curve. In brief, samples were diluted up to 1:32 with the provided kit buffer and transferred to a 96-well microplate and mixed with an equal volume of the provided reaction mix (lactate oxidase and lactate probe). After 30 min of incubation at room temperature, the microplate was read at 570 nm on a Spectramax 340 (Molecular Devices, Sunnyvale, CA, USA). Lower limit of quantification for L(+)-lactate was 0.02 mmol/L.

4.3.2 Immunoassay

Prostaglandin E_2 (PGE_2)

PGE2 levels in the synovial fluid samples were analyzed using a PGE2 radioimmunoassay KIT (RPA 530, GE Healthcare, Uppsala, Sweden). The assay was performed according to the manufacturer's instructions, with the exception that the volume of the oximation reagent was adapted to the viscosity of the samples.

Cytokines and Chemokines

All assays were performed using the electro chemiluminiscence (ECL) technique. 96-well plates, pre-coated with antibodies towards the analytes of interest, were blocked with the provided diluent for 30 min, thereafter plasma/serum or synovial fluid samples, diluted 1:2 or 1:4 respectively in assay diluents, were added to the wells and the plates were incubated for 2 h. Following washing of the plates, MSD Sulfo TAG secondary antibody mixtures were added and the plates were incubated for an additional 1.5 hrs. All incubations were performed at room-temperature. After a final washing step, read buffer (2X) was added and the plates analyzed on a SECTOR Imager (SI6000, MesoScale Discovery).

In house developed immunoassay for rat NGF

An immunoassay for analysis of rat NGF was developed using electro chemiluminescence (ECL) technique. Plates were coated with Rat beta-NGF antibody (Cat No AF-556-NA),

blocked with 3% BSA for 30 minutes and subsequently washed. Recombinant Rat beta-NGF (Cat No 556-NG-100/CF) was used as a calibrator. Synovial fluid samples, diluted 1:4 in assay diluents, were added to the wells and the plates were incubated for 2 h. Following washing of the plates, Rat beta-NGF biotinylated antibody (Cat No BAF556), added with MSD Sulfo TAG, was dispensed to all wells and the plates above. Lower limit of quantification was 9.77 pg/mL.

4.4 Statistics

To compare the treatment groups in **Paper I**, statistical evaluation of PGE_2 and lactate levels was performed by using an unpaired t-test, Graph Pad Prism 4 software (GraphPad Software, La Jolla, CA, USA). The effect of the anti-inflammatory drugs on the degree of inflammation was evaluated by using Kruskal–Wallis one way analysis of variance (ANOVA) on ranks (non-parametric data) followed by Dunnett's test to compare the vehicle-treated group to the treated groups. Statistical significance was set at $p<0.05$. Results are presented as mean ± SEM (**Paper I**).

Time course of inflammatory biomarkers from the animal studies (FCA and/or MIA injected rats) were presented as mean level together with 95% confidence interval for each time point (**Paper II and V**). No formal hypotheses were tested in the FCA evaluations in Paper II. All statistical analyses in **Paper II** were performed using either GraphPad Prism version 6.00 or SAS/STAT version 9.3 of the SAS System for Windows (SAS Institute, Cary, NC, USA). In the MIA study, the response variables were evaluated in a regression model using study group (time point) as the exploratory variable. Before the model fit, a root transformation was applied to the response variables. The assumption of approximately normally distributed residuals in the statistical modelling process was thereby better fulfilled and the variance stabilized. In the humans, correlation between the two blood matrices as well as between the blood and SF were tested by calculating Pearson's correlation coefficient together with the corresponding P-value. The difference between the two blood matrixes groups were compared in an ordinary t-test. Both the correlations and t-tests were based on log-transformed data. The Mann-Whitney U-test was utilized to compare patients and controls with respect to cytokine levels.

In **Paper III** Friedman test with Dunn's multiple comparison test was used to analyze cytokine measurements for individual groups over time. Kruskal-Wallis test with Dunn's multiple comparison tests was used to analyze cytokine measurements for three groups at one time-point. Linear regression analysis was used to determine correlations between axonal rescue and cytokine measurements, as well as between autofluorescence measurements of cord and spleen to MIP-3α measurements after Imatinib treatment.

In **Paper IV** differences in IL-6 between freeze/thaw cycles were analyzed using the Mann–Whitney *U* test. All values were reported as mean ± SEM. Reported *p* values are 2-sided. In this paper authors performed the analyses using SPSS Statistics version 20.0 (IBM Inc.) or Prism version 6.0 (GraphPad Software Inc.)

5 RESULTS

Rats subjected to either Carrageenan (Cg) or Freund`s Complete Adjuvant (FCA) developed a time-dependent inflammation at the site of injection, the ipsilateral ankle joint, whereas no inflammation was observed at the contralateral joint. Joint swelling was measured by visual scoring (Paper I) or by using a three-button digital caliper (**Papers II and V**) to confirm inflammation.

In **Paper I**, an intra-articular injection of either Cg or FCA caused an increase of prostaglandin E_2 (PGE_2) and L(+)-lactate in synovial fluid from the inflamed joint. Both markers showed a similar time course pattern, with a peak at 8 hrs in the carrageenan model. This observation is in contrast to the FCA model of inflammation where L(+)-lactate levels increased more rapidly (max at 24 hrs) as compared to PGE_2 (max at 48-72 hrs). Both treatments, naproxen or rofecoxib, significantly decreased PGE2 levels but did not affect L(+)-lactate concentration in synovial fluid (Table II a).

In **Paper II** levels of monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 3α (MIP-3α), KC/GRO (IL-8 in humans), IL-1β, IL-6 and TNF were analyzed in synovial fluid, serum and EDTA plasma from two animal model Freund´s Complete Adjuvant (FCA) and monoiodoacetate (MIA), and from human subjects with osteoarthritis (OA). The aim was to identify soluble biomarkers of ongoing inflammation in the joint from a translational viewpoint.

Levels of L(+)-lactate were measured in all joint fluids. In line with the findings of Paper I, we could confirm the same pattern and levels of L(+)-lactate in the FCA model and here the levels correlated with MIP-3α levels, with a peak at 24 hrs. Levels of L(+)-lactate differed in magnitude as well as in temporal pattern between the two animal models, the MIA model expressed a biphasic pattern and levels were still increased at 35 days post-injection. Five individuals in the OA group had slightly to moderately higher L(+)-lactate levels in synovial fluid and one had extremely high levels compared to the lowest level of L(+)-lactate in this group. No normal/non-inflamed human synovial fluid was available (Table II b).

Elevated levels of chemokines/cytokines were observed in the synovial fluid of monoarthritic animals as compared to control groups, although with considerably lower magnitude in the MIA groups, which also demonstrated a biphasic pattern. Levels of KC/GRO and MIP-3α in serum from the FCA model and IL-6 in the MIA model followed a similar pattern to that observed in synovial fluid. In serum samples from OA individuals, only MIP-3α correlated significantly with levels in synovial fluid (Table II b).

In **Paper III** we aimed to determine if there were changes in inflammatory biomarkers in serum during the time of treatment that could be used as a biomarker to indicate an effect of a drug treatment. Imatinib was given four hrs following a moderately induced spinal cord injury (SCI) and then twice daily for seven days. For controls, we used rats that underwent

SCI without drug, as well as sham-injured and uninjured (naïve) animals with or without drug. Both MCP-1 and MIP-3α in serum were elevated at day 1 for all the surgery groups (injured and sham-injured) as compared to basal levels (uninjured). KC/GRO showed a different pattern at day 1, with a significant increase in the sham-injured group as compared to injured and uninjured groups, but with a decline to basal levels at day 7 post-sham injury. However, both MCP-1 and MIP-3α were further increased at 7 days following injury in the imatinib group in comparison with the other groups, in which levels remained similar or lower throughout the study (Table II c), Furthermore, each individual animal in the treatment group showed a significant increase of the three biomarkers MCP-1, MIP-3α and KC/GRO in serum as compared to their starting value at day 1 following injury, indicating that an individual can be its own control.

Nerve Growth Factor (NGF) is released following tissue injury. In **Paper V**, one of the aims was to assess the presence of NGF in synovial fluid from rats exposed to FCA. A method for the measurements of rat NGF was successfully developed based on electro chemiluminescence technology. The level of NGF in synovial fluid from rats injected with FCA was significantly increased, reaching a maximum at days one and two and thereafter declining but was still significantly increased at days 5 and 10 as compared to saline injected animals (Table II d).

Methodological aspects

All assays used in this thesis are based on electro chemiluminescence (ECL) technology with the exception the assay for L(+)-lactate which is a colorimetric assay. Given the very low levels of analytes, some of the biomarker results in Papers I, II, III and V are close to the low limit of quantification (LOQ), meaning that levels below LOQ cannot be accurately determined, but are evaluated as similar to LOQ, or close to, LOQ. All assays underwent a normal validation before measurement of the study samples. Interestingly, in paper II, MCP-1 and KC/GRO (IL-8 in humans) showed different ranges and dynamics in serum and plasma in both the FCA model and in humans, with considerable lower levels in plasma. This observation has implications for the practical application of these assays in broader contexts.

In **Paper IV**, we refer to low limit of detection (LOD), meaning that, as above, some values are estimated to be low or close to LOD. Due to limitations in sample volumes and frequent sample handling, it is of importance to check if the analyte of interest (IL-6 in cerebrospinal fluid) still shows reliable results at that low level due to sample age and freeze/thaw cycling. Therefore a stability test was performed to assess the effects of repeated thawing/freezing cycles of IL-6. No significant difference in IL-6 concentrations was observed between CSF samples, taken from -70° C, that was thawed once or after two thawing/freezing cycles. This suggests that the assay is indeed measuring IL-6 levels close to LOD.

5.1 **TABLE II.** Summary of results.

a)

Paper I	**Drug admin. post induction (hrs)**	**Synovial fluid collected post drug admin. (hrs)**	**PGE_2**	**L(+)-lactate**
Cg			↑	↑
Cg + Naproxen	2	3	***↓	↑
Cg + Rofecoxib	2	3	***↓	↑
FCA			↑	↑
FCA + Naproxen	48	3	***↓	↑
FCA + Rofecoxib	48	3	***↓	↑

↑ = increased levels compared to its own control group. ***P<0.001.
Cg = Carrageenan, FCA = Freund´s Complete Adjuvant

b)

Paper II	*Synovial fluid*							
	Time points	**IL-6**	**KC/GRO IL-8**	**MCP-1**	**MIP-3α**	**IL-1β**	**TNF**	**L(+)-Lactate**
FCA	5-48 hrs	↑	↑	↑	↑	↑	↑	↑
MIA	24-48 hrs, and day 21, 28	↑ a	↑ a	↑ a	↑ a	–	–	↑ a
OA individuals		↑ c	↑ c	↑	↑ c	– d	– d	↑ b
		Correlation synovial fluid /serum						N/A
FCA		no	yes	no	yes	no	no	
MIA		yes	no	no	no	no	no	
OA individuals		no	no	no	yes	no	no	
		Differences in serum levels						
OA/healthy individuals		*yes	no	no	no	no	no	
		Different distribution of serum and EDTA plasma						
FCA		no	yes	yes	no	no	no	
OA		no	yes	yes	no	no	no	

↑ = increased levels compared to its own control group (FCA and MIA), **--** = levels below or close to limit of quantification, a = moderately increased levels compared to FCA, b = increased slight to extremely, c = one individual with extreme levels, d = all at the same level with one exception. *P<0.05 N/A = not applicable

c)

Paper III	*Serum levels compared to baseline levels*								
	MCP-1			**MIP-3α**			**KC/GRO**		
	time points (days)								
	1	3	7	1	3	7	1	3	7
Uninjured	baseline								
Uninjured + Imatinib (4 h)			↑			↑			↑
Sham injured	*** ↑	–	* ↑	** ↑	–	–	*** ↑	–	↓
Spinal cord injury	** ↑	–	** ↑	–	–	–	–	–	–
Spinal cord injury + Imatinib (4 h)	–	–	*** ↑ e	–	↓	*** ↑ e	–	–	** ↑ e

↑ = increased levels compared to baseline levels in uninjured rats.

- = no significant differences between groups of animals per time point. *P<0.05, **P<0.01, ***P<0.001.

↑ e = significantly increased compared to the injured group (spinal cord injury) that did not receive imatinib as well as to the sham group.

A multiplex (10-plex) was used for the measurements of cytokines and chemokines and no significant differences was found for INF-γ, IL-1β, TNF, IL-6, IL-2, IL-4, IL-5, IL-10 and IL-13, although IL-6 differs slightly between the surgery models. Serum levels of MCP-1, MIP-3α, and KC/GRO were also affected by imatinib alone and induced a similar temporal pattern of increase as in injured imatinib treated animals, although the levels were not as high as in imatinib treated animals with spinal cord injury.

d)

Paper V	*NGF in synovial fluid*			
	timepoints (days)			
	1	2	5	10
Saline	baseline	N/A	N/A	baseline
FCA	* ↑	* ↑	* ↑	* ↑

↑ = increased levels compared to baseline levels at day 1 and 10. *P<0.05. N/A = not applicable.

No significant difference between baseline levels at day 1 and 10 in the saline injected groups.

6 DISCUSSION

The present study has focused on biochemical biomarkers, with an emphasis on inflammatory markers at the site of inflammation and in peripheral blood. In experimental trials of inflammation, biomarkers should reflect ongoing inflammation, the progression of inflammation and, depending on the designed purpose, should also reflect response to pharmaceutical treatment. Furthermore, knowledge as to which biomarkers covary between animal models and human disease will enable translation of preclinical data, and in the most optimal case, identify targets for disease and symptom therapies. The work presented in this study has addressed the optimisation of all these aspects.

The development of a biochemical biomarker is a process consisting conceptually of five phases (Lee et al, 2009).The development of biomarkers is performed mostly in the initial discovery phase but also during the demonstration phase in the case of novel biomarkers. The discovery phase includes not only the measurement of an exploratory biomarker in animal studies or cell studies, but also the use of an appropriate assay. Since the majority of biochemical biomarkers are proteins, measured in biological matrices such as blood, synovial fluid or cerebrospinal fluid, immunoassays are the most commonly used method for quantification of the analytes. Most assays are commercially available, as RUO kits (for research use only, discovery-grade) (Bowsher et al, 2012, Lee et al, 2009). While these kits may have limitations in some cases, there are also advantages e.g, the kits are ready to use, simple to use and also cheaper in comparison with an in-house developed assay. Even with RUO kits, it is still necessary to perform a proper validation, before use of the intended application, to ensure that it measures the right biomarker in the right matrices at a correct level.

Different animal models are used to induce pathology in rodents that resembles human joint disease. Common rat models of joint inflammation are the carrageenan model, the Freund´s complete adjuvant (FCA) model and the monoiodo acetate (MIA) model. The monoarthritis is induced by an intra-articular injection of the agent to the joint but the degree of the developed inflammation may differ within the group due to technical issues or to individual variability. In the acute phase of inflammation neutrophils infiltrate the synovial membrane and articular cavity. These inflammatory cells are dependent on glucose as an energy source and glycolysis contributes to lactate (the end product of glycolysis) accumulation in the presence of oxygen, especially under inflammatory conditions (Samuvel et al, 2009) Therefore we included assessment of L(+)-lactate, as a possible marker in synovial fluid from the affected joints as well as measuring established inflammatory biomarker, prostaglandin E_2 (PGE_2).
A prerequisite for the use of lactate as a biochemical biomarker was that it should provide information about the ongoing inflammation and preferably without interfering with the

target. In **Paper I** we could demonstrate significant decrease of PGE_2 in synovial fluid following treatment with two COX-inhibitors, naproxen and rofecoxib, despite the drugs having limited efficacy on overall joint swelling. We could also demonstrate that L(+)-lactate levels were unaffected by COX-inhibitors, indicating that L(+)-lactate can be a useful marker of experimentally induced inflammation especially for monitoring the non-cox-inhibitor sensitive cascade. Furthermore, lactate may therefore have direct utility as a pharmacodynamic marker in drug development for inflammatory joint diseases.

In **Paper II** the main purpose was to identify possible soluble biochemical biomarkers from a translational viewpoint, meaning that we compared findings/biomarkers from established animal models with those from patients with osteoarthritis.
The FCA model has mainly been used to study the pathology and/or symptoms of rheumatoid arthritis, such as joint pain, while the MIA model is used for studies of the pathology of osteoarthritis (Muir et al, 1982, Guzman et al 2003, Bendele et al, 2001). The MIA model is not totally accepted as a model of human OA pathology, since some regard this model as mainly exhibiting painful behavior, but the model primarily induces cartilage degradation, osteophyte formation and mild synovitis, and thus reflects many of the hallmarks of OA.

Synovial fluid from patients can be taken in two ways, either from outpatient clinic volunteers who are undergoing drug treatment, or taken from patients in the late stage of disease during joint replacement surgery. Results from patients in the former group, presented in Paper II, may differ depending on the sampling technique and may not be directly comparable with results from patients in the latter group. All human subjects in our study had ongoing drug treatment (NSAIDs or prednisolone), which potentially could influence the synthesis of cytokines such as IL-6, TNF and IL-8 (Gallelli et al 2013, Youssef et al, 1997, Young et al, 2001).

In synovial fluids, all tested biomarkers were observed in the FCA model, but only IL-6, KC/GRO, MCP-1 and MIP-3α were increased (not IL-1β and TNF), although at a lower magnitude, in the MIA model, suggesting that the underlying pathology in these two models is significantly different. Both models also had different systemic inflammatory profiles since only IL-6 in MIA serum followed the pattern of IL-6 in synovial fluid, whereas in the FCA model IL-6, KC/GRO and MIP-3α levels were all similarly elevated in both matrices. In five of the six OA individuals we observed a wide range of levels for all biomarkers, except IL-1β and TNF, in either synovial fluid or serum. One individual (donor 1) showed extremely high levels of all markers in synovial fluid, but which were not that markedly increased in serum. However, one of the aims of this study was to investigate whether markers in blood reflect the levels in synovial fluid, which was true for MIP-3α in humans as also seen in the FCA model. Nevertheless, we could not observe any significant difference when comparing levels of MIP-3α in the OA group with the control group, therefore this chemokine cannot be regarded as a biochemical biomarker in OA individuals despite being correlated between

serum and synovial fluid. Interestingly, we observed two common findings between the MIA model and in humans, namely the lack of IL-1β and TNF in synovial and serum (in five out of six patients), and the presence of elevated levels of IL-6 in joint and serum. However, we did not find a positive correlation between IL-6 in synovial fluid and serum from the OA individuals but as indicated in the MIA model, where serum IL-6 levels followed synovial fluid with a lag time, it is possible that the IL-6 levels change over time, implicating different states of disease progression.

Elevated levels of IL-1β and TNF have previously been reported mainly in RA patients and more recently others have concluded that IL-1β is not consistently elevated in the OA joints (Scanzello et al, 2012) On the other hand, reports indicate that IL-1β and TNF in articular cells amplify osteoarthritis by inducing the production of several inflammatory biomarkers and mediators including IL-6, IL-8 and MCP-1 (Kapoor et al, 2011, Brenner et al, 2004). It could also be a question of time, as an example, if IL-1β precedes IL-6, it is possible that the phase of IL-1β and TNF involvement had passed the time for detection of these markers both in the MIA model and in humans where the disease progression might have advanced to a low IL-1β and TNF state. Recently, it has been shown that the limited success with anti-IL-1 drugs as disease modifying drugs for OA could be explained by the varying degree of IL-1β involvement in OA pathology (Kopf M et al, 2010, Jotanovic et al, 2012). X. Chevalier reported 2009 that anakinra (anti-IL-1) was hypothesized to counter symptoms of pain and stiffness or changes in cartilage metabolism that may be related to excess IL-1 or an IL-1–related effect but, in their study, there were no improvements in knee pain, function, stiffness, or cartilage turnover.
Finally, **in Paper II** we predicted, due to findings in paper I, that increased levels of L(+)-lactate would be found in the affected joints and as expected, L(+)-lactate was readily detectable, correlating (in most cases) with the levels of cytokines and chemokines and thereby also indicating the presence of invading cells into the joint cavity. Overall, this suggests that a general on-going inflammatory process in the joints is being reflected by increased levels of L(+)-lactate.

In **Paper III** one of the main aims was to determine if there were changes in inflammatory biomarkers in serum during the time of treatment that could be used as a biomarker that indicates an effect of the drug imatinib in rats subjected to spinal cord injury. In addition to the classical markers, INF-γ, IL-1β, TNF, IL-6, KC/GRO, IL-2, IL-4, IL-5, IL-10 and IL-13, measurable with a 10-plex immunoassay plate (Mesoscale Discovery) we analyzed the MCP-1 (monocyte chemoattractant protein-1) and MIP-3α (macrophage inflammatory protein). Both MIP-3α and MCP-1 are known to have chemotactic properties and are associated with macrophages, and as shown in Paper II, both markers could readily be measured in both rat and human serum, with or without a challenge. MCP-1 has previously been shown to increase in patients with cancer receiving the drug imatinib (Hayashi et al, 2012) , but in general, no biomarkers have been explored or used in previous clinical trials with treatments for spinal cord injury. In **Paper III**, we could demonstrate that levels of MCP-1

and MIP-3α were significantly increased in serum, from day 1 to day 7 following injury in the imatinib group. These two markers were also elevated similarly in serum from uninjured rats that received imatinib and where MIP-3α proved to be more robust (less variation and higher significance) as compared to MCP-1. KC/GRO showed a slightly different pattern of levels throughout the study, but was significantly increased as well by day 7 following injury and treatment.
These results indicate that we have found a time point (7 days) when the increase of MCP-1, MIP-3α and KC/GRO can be usefully monitored as a biomarker of treatment effect, and if comparing with levels from day one the SCI individual could be its own control. It is plausible that our findings will translate to humans. Finally, it might be useful to use all these three biomarkers in combination to evaluate the most robust temporal response and thereby distinguish responders from non-responders to imatinib.

In **Paper I and II** we demonstrate findings regarding several inflammatory biomarkers produced as a result of induced inflammation with Freund´s Complete Adjuvant, where prostaglandin E_2, not only known to be a well-established biomarker for inflammation, is also known to promote inflammatory pain by sensitizing afferent nerve endings. It is also known that nerve growth factor (NGF) is released following tissue injury and experimental data indicates that NGF is important in initiating and maintaining persistent pain associated with inflammation. In **Paper V** we aimed to test whether blockade of NGF, TRPV1 and COX activity generated a similar anti-nociceptive profile in rats as previously demonstrated in humans (Lane et al, 2010 Spiering et al, 2013). Firstly, we wanted to test whether NGF is released into the synovial fluid in the FCA model. No specific rat NGF kit is available commercially so for this we developed an immunoassay using electro chemiluminescence technology. We could successfully detect NGF in rat joints fluid subsequent to induction with Freund`s complete adjuvant. NGF in synovial fluid following saline injection were unchanged from one day to 10 days whereas induction with FCA significantly increased the levels of NGF suggesting that this model can be used to demonstrate efficacy of several treatments related to increased levels of NGF including anti-NGF treatment itself.

Methodology

Normally a validation of kits is performed in advance of analysis of study samples, and in some cases the validation has to be extended with more dedicated stability tests due to the expectations of the study or due to limitations in study samples such as age, volume and frequent sample handling.It is important to evaluate which matrix, serum or plasma, is most appropriate to use for detection of the biochemical markers especially if they are sensitive to clotting factors. In **Paper II** we found differential distribution of markers, KC/GRO and MCP-1 between serum and EDTA plasma preparations from the same animal, and we also observed a similar difference in the distribution of IL-8 (KC/GRO in rats) and MCP-1 between serum and EDTA plasma within the same human These findings have made us aware that

the right matrix has to be used for any given specific analyte. This finding is of great general utility in the field.

In **Paper IV** all samples had been thawed more than once and levels were therefore expected to be low or maybe not even detectable at all. Only two markers, IL-6 and IL-8, reached levels above detection limit (the lower definition of sensitivity). If too many sample levels are below limit of quantification but above limit of detection it is important to check that no other parameters are affecting the levels, such as several freeze-thaw cycles which is known to affect the concentration of cytokines. Such an effect must be checked with stability tests for the marker of interest using a more sensitive assay. Our choice was an IL-6 single-plex in contrast to the 10-plex that was used for the original study samples. However, no significant difference in IL-6 concentration was observed in cerebro spinal fluid (CSF) samples that had been thawed once or twice, and levels were found to be above the detection limit in all samples. We conclude that IL-6 in CSF is not sensitive to this number of freezing /thawing cycles.

Lastly, critical aspects for the development of immuno assays are the choice of and use conditions for capture and detection antibodies. In **Paper V** we reported the successful development of an assay for the measurement of rat NGF in synovial fluid. The included antibodies were tested at several concentrations to find the optimal limit of quantification and the lowest possible background values, but were not tested for related proteins. To even further improve the assay, it could be tested against other proteins to assure the specificity for NGF, but the antibodies used are stated to be specific due to the manufacturer (R&D Systems).

In conclusion, in experimental trials of inflammation there is a need for stable and reliable biochemical biomarkers that reflect the biological process and specific treatment effects. While we found increased levels of inflammatory biomarkers in joint fluid and blood, we found difficulties to identify a single systemic biochemical biomarker to illustrate an ongoing local inflammatory process in osteoarthritis. Furthermore, we conclude that inflammatory biomarkers, in either joint fluid or serum, could be used to evaluate the efficacy of specific drugs. Lastly, we found that some biomarkers have different ranges and dynamics in serum and plasma in both the animal models and in humans. We conclude that the usage of biochemical biomarkers has wide application, and may be used as a complementary tool to other readouts, such as symptoms, for the analysis of inflammatory conditions.

7 GENERAL CONCLUSIONS

Biological aspects

Paper I

L(+)-lactate can be used as an additional biochemical biomarker of inflammatory processes in rats.

- Both biomarkers, L(+)-lactate and PGE_2 increased following inflammation challenges.
- Treatment with COX-inhibitors resulted in a significant decrease of PGE_2 whilst L(+)-lactate was unaffected.
- Inhibition of PGE_2 synthesis resulted in only partial acute anti-inflammatory properties.

Paper II

Elevated levels of inflammatory biomarkers are present in the arthritic joints in rat and human.

- The presence of pro-inflammatory markers in the joint fluids indicates ongoing inflammation.
- L(+)-lactate might be used as a biomarker for ongoing general inflammation in the human OA joint.

Biomarkers in blood reflected the ongoing inflammation in joints in osteoarthritic individuals and in arthritis models but no specific translational biomarker between the OA patients and animal models was found.

- More biochemical biomarker work needs to be done regarding patients with osteoarthritis.
- There is a need to identify a credible translational rodent model of osteoarthritis.

Paper III

MIP-3α and MCP-1 in serum can be used as biomarkers following imatinib treatment.

- Chemokine levels in serum on an individual basis may serve as candidate biomarkers for the effect of imatinib.
- Chemokine levels in serum might also be used to stratify patients, such as to distinguish imatinib responders from non-responders.

Paper V

Nerve growth factor (NGF) is a product of FCA induced inflammation in rats.

- A successful method for the evaluation of rat NGF was developed.
- FCA induced monoarthritis might be a relevant model when testing drugs targeting the NGF/TrkA pathway.

Methodological aspects

Paper II

The right matrix has to be used for any given specific biomarker.

- Some biomarkers (MCP-1 and KC/GRO, IL-8) have differential distribution and dynamic range of movement in serum and plasma.

Paper IV

A proper stability test provides information about the possible use old samples.

- IL-6 in human cerebrospinal fluid can be measured following repeated thaw/freeze cycles

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Biokemiska biomarkörer är små komponenter, naturligt förekommande eller experimentellt framkallade, som är mätbara i kroppsvätskor och som kan ge information om hur kroppen mår. Biomarkörer kan vara ett ”verktyg” för att beskriva sjukdomsprocesser eller för att bedöma hur individer svarar på farmakologisk behandling. Fokus i denna avhandling är att studera inflammatoriska markörer, så kallade mediatorer, kemokiner och cytokiner som lockar immunceller från blodet till platsen för skada och som reglerar förloppet av den inflammatoriska processen vid platsen för inflammation, exempelvis en knäled. Vi ville också ta reda på om det är möjligt att mäta markörer i blod som speglar inflammationsförloppet i en kroppsdel. Dessa biomarkörer mäter man med speciella analyser där antikroppar är den viktigaste komponenten.

I den första studien använde vi två etablerade djurmodeller, Freund's Complete Adjuvant (FCA) och karragenan för att skapa, inducera, experimentell inflammation i ankelled hos råtta och för att bevisa att inflammationen pågick i leden mättes ledsvullnad. Vi gav djuren två cyklooxygenas (COX) hämmare, naproxen och rofecoxib, och kunde påvisa en signifikant minskning av prostaglandin E_2 (PGE_2), som är en produkt av inflammation och så kallad mediator, i ledvätska efter behandling trots att drogerna har begränsad effekt på den totala ledsvullnaden. L(+)-laktat, som är slutprodukten av sockeromsättningen i cellerna och som återspeglar cellaktivitet, kan därför vara en lämplig indikator på experimentella inflammatoriska tillstånd. Vi fann att L (+)-laktatnivåer var opåverkade av COX-hämmare i denna studie, vilket tyder på att L (+)-laktat kan användas som en biokemisk biomarkör för pågående inflammation hos individer under behandling med COX-hämmare. I nästa studie mätte vi nivåer av L(+)–laktat, såväl som kemokiner och cytokiner, i ledvätska från patienter med artros och från en ytterligare djurmodell, monoiodoacetate (MIA), vilken främst inducerar brosknedbrytning, osteofytbildning och lindrig synovit. MIA producerade distinkta inflammatoriska biomarkörer i ett bifasiskt mönster men med betydande lägre magnitud (nivåer) än FCA modellen, vilket tyder på att den underliggande patologin i dessa två modeller är betydligt annorlunda. Som väntat, var L(+)- laktat mätbar i ledvätska i MIA-modellen och hos människa, som korrelerade med nivåerna av cytokiner och kemokiner vilket indikerade närvaro av inflammatoriska celler i leden. Vi observerade även två gemensamma fynd mellan MIA modellen och hos människa, närvaron av IL-6 i leden och serum samt avsaknaden av IL-1β och TNF i samma matriser. Trots detta fann vi det svårt att identifiera en systemisk biokemisk biomarkör som kunde illustrera en pågående lokal inflammatorisk process hos patienter med artros. Vi kunde inte heller identifiera en specifik translationell (överförbar) biomarkör mellan djurmodeller och individer med artros.

I den tredje studien, hos råttor som utsatts för ryggmärgsskada, var de viktigaste resultaten förändringar av inflammatoriska biomarkörer i serum under tiden för behandling med läkemedlet imatinib, en tyrosinkinashämmare. Även om serumnivåer av de kemotaktiska markörerna MCP-1 och MIP-3α var förhöjda vid dag 1 efter skada eller simulerad skada, förblev nivåerna likartade eller lägre under hela studien upp till 7 dagar. MCP-1 och MIP-3α

var dock ytterligare förhöjt 7 dagar efter inducerad skada i imatinibgruppen. Det är troligt att våra resultat kommer att kunna översättas till människa och att den ryggmärgsskadade individen kan vara sin egen kontroll vid jämförelse med värden vid skadans början.

En ytterligare faktor, förutom de som tidigare nämnts som frisätts efter vävnadsskada är nervtillväxtfaktorer (NGF). Flera studier har visat att under den inflammatoriska processen finns det en lokal ökning av NGF vid inflammationsstället och NGF kan vara relaterad till uppkomst och upprätthållande av inflammmatorisk smärta. Vi behövde därför veta om NGF var mätbart i ledvätska hos djur med FCA inducerad inflammation. Vanligtvis finns det ”analyskit” kommersiellt tillgängliga för de flesta analyser men i detta fall fanns endast de enskilda ingående kemikalerna att köpa, vilket betyder att forskaren måste utveckla/skapa en analysmetod för dedikerad frågeställning. I den sista studien rapporterade vi om en framgångsrik utveckling av en analysmetod för mätning av råttNGF i ledvätska hos råttor som exponerats för FCA och som i sin tur möjliggjorde fortsatta studier där förekomsten av NGF i ledvätska var viktig information.

En slutsats från dessa studier är att biokemiska markörer har ett brett användningsområde, till exempel för att användas som kompletterande verktyg till klinisk diagnostik, för identifiering av inflammatoriska tillstånd.

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